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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12P 19/34, C12Q 1/68

A1

(11) International Publication Number: WO 93/18177

(43) International Publication Date: 16 September 1993 (16.09.93)

(21) International Application Number: PCT/US93/02259

(22) International Filing Date: 11 March 1993 (11.03.93)

(30) Priority data:
07/850,703 13 March 1992 (13.03.92) US

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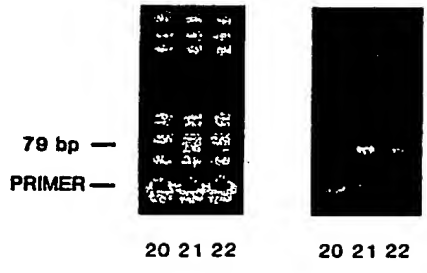
(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

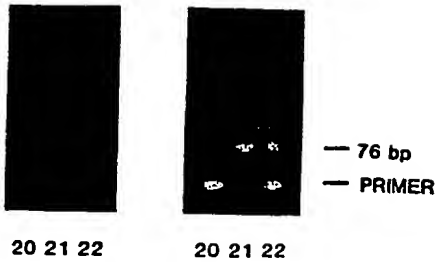
Published
With international search report.

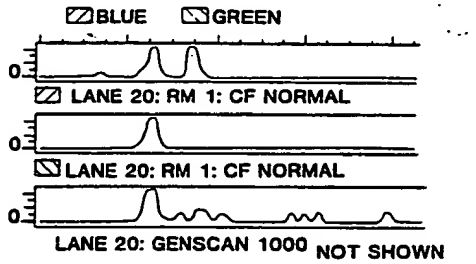
(54) Title: DIAGNOSIS OF CYSTIC FIBROSIS USING ALLELE SPECIFIC MULTIPLEX POLYMERASE CHAIN REACTIONS

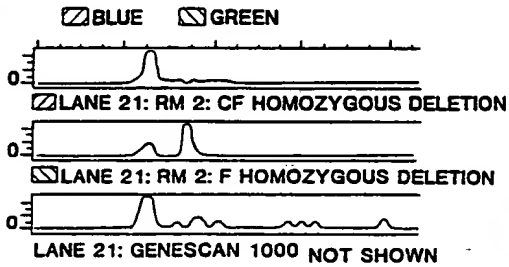
(57) Abstract

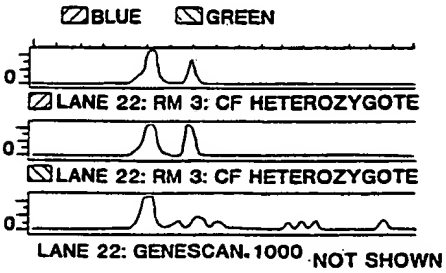
The present invention provides methods of diagnosing diseases such as cystic fibrosis using an allele specific multiplex polymerase chain reaction system as illustrated by the electrophoresis gels and densitometry tracings. In addition, kits useful for diagnosing diseases such as cystic fibrosis are provided.











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DIAGNOSIS OF CYSTIC FIBROSIS USING ALLELE SPECIFIC MULTIPLEX POLYMERASE CHAIN REACTIONS

CROSS REFERENCE TO RELATED APPLICATIONS

This application is related to U.S. application Serial No. 850,704 filed March 13, 1993 entitled "Diagnosis of β-Thalassemia Using a Multiplex Amplification Refractory Mutation System", assigned to the assignee of the present application and incorporated by reference in its entirety. (Attorney Docket No. CH-0225)

FIELD OF THE INVENTION

This invention is directed to the diagnosis of cystic fibrosis using a novel multiplex allele-specific polymerase chain reaction system.

BACKGROUND OF THE INVENTION

Cystic fibrosis (CF) is the most common lethal autosomal recessive disease in Caucasians affecting approximately 1 in 2000 newborns with a carrier frequency of about 1 in 25 individuals. More than 120 different mutations have been reported in the CFTR gene; Kerem, et al., *Science* 245: 1073-1080 (1989); Zielenski, et al., *Genomics* 10: 214-228 (1991); Dean, et al., *Cell* 61: 863-870 (1990); Cutting, et al., *Nature* 364: 366-369 (1990); Kerem, et al. *Proc. Natl. Acad. Sci. U.S.A.* 87: 8447-8451 (1990) and Cystic Fibrosis Genetic Analysis Consortium, *Am. J. Hum. Genet.* 47: 354-359 (1990); with the ΔF508 mutation, a 3 basepair (bp) deletion in the first nucleotide binding fold (NBF), representing the most common allele in Caucasians. The frequency of this deletion

varies substantially among different populations; ΔF508 is found in approximately 89% of all CF chromosomes in Danish, 71% in French Canadian, 49% in Spanish and 43% in Italian patients. Ng, I. et al., *Hum. Gent.* 87: 613-617 (1991). Five mutations have been identified (ΔF508, G551D, R553X, G542X and N1303K) which account for 84% of all Caucasian CF chromosomes. Thus focus has been centered on these mutations in order to achieve accurate diagnosis.

Much consideration has been given to the issue of the advisability of widespread screening for CF. Effective tests should be able to detect multiple mutations, should be cost effective, rapid and accurate. Of utmost importance is the ability to handle large number of samples, preferably by automated methods. Furthermore, methodologies should be simple enough to be used in clinical laboratories.

A wide variety of strategies and techniques are currently available to detect CF mutations. A two tube allele-specific PCR test was recently described for the ΔF508 mutation which greatly simplifies detection of this allele Ballabio, A., et al., *Nature* 243: 220 (1990). In addition, a single tube multiplex allele specific PCR test using two different dye-tagged fluorescent primers for detection of the ΔF508 mutation has also been suggested which permits carrier detection in a single lane of a gel. Ballabio, A., supra. Unfortunately, these methods are designed to identify only the ΔF508 deletion, which accounts for only about 75% of mutant alleles. In order to be effective for widespread and accurate screening for CF, tests must be developed which can diagnose multiple mutant alleles, thus increasing the likelihood that CF will be diagnosed.

Multiplex PCR has also been used for simultaneous amplification of multiple target sequences, permitting mutant allele scanning using two lanes of an agarose gel. This strategy involves appropriate choice of primer pairs so that PCR fragments (either normal or mutant) are generated of different size which can be easily resolved by comparison of samples run in parallel lanes of a gel. In males, deletional

forms of X-linked diseases such as Lesch-Nyhan syndrome and Duchenne muscular dystrophy are immediately obvious with this procedure, since missing exons are readily apparent in the amplification pattern. Gibbs, et al., *Genomics* 7: 235-244 (1990); Chamberlain, J.S., et al., *Nucleic Acid Research* 16: 11141-11156 (1988); Beggs, et al., *Hum. Genet.* 86: 45-48 (1990). Such a diagnostic tool, effective for simultaneously detecting multiple allelic mutations, quickly and accurately is greatly desired for the detection of cystic fibrosis.

SUMMARY OF THE INVENTION

Methods of diagnosing CF which are effective for simultaneously detecting multiple allelic mutations quickly and accurately are provided by the present invention. The methods of the present invention comprise the steps of obtaining genomic DNA from a patient suspected of carrying a genetic mutation characteristic of cystic fibrosis and selecting at least two primer sets for detecting the normal and mutant allele characteristic of cystic fibrosis. Each primer set is comprised of two primer pairs, a first primer pair comprising a specific primer for a normal allele, and a second primer pair comprising a specific primer for a mutant allele. Each pair further comprises a common primer. A polymerase chain reaction is performed in accordance with methods of the present invention using said genomic DNA and said at least two primer sets whereby primer pairs comprising a specific primer for a normal allele are used simultaneously and primer pairs comprising a specific primer for a mutant allele are used simultaneously. Two or more polymerase chain reaction products are detected whereby the detection of a polymerase chain reaction product of a specific primer for a mutant allele indicates the likelihood that said patient carries a mutation characteristic of the phenotype cystic fibrosis. In some embodiments of the present invention, each specific primer is differentially labeled. In still other embodiments of the present invention the genomic DNA and all differentially labeled primer sets are used simultaneously to perform the

polymerase chain reaction.

In another embodiment of the present invention kits are provided comprising four dNTPs and at least two primer sets selected from the group consisting of

CF-W1468 GGCACCATTAAAGAAAATATCATCTT (SEQ ID NO: 1),
Δf508 GGCACCATTAAAGAAAATATCATTGG (SEQ ID NO: 2), and
CF-508RP TAGTGTGAAGGGTTCATATGCATAAT (SEQ ID NO: 3);

R553X-N CTAAAGAAATTCTTGCTCG (SEQ ID NO: 4),
R553X-M CTAAAGAAATTCTTGCTCA (SEQ ID NO: 5), and
IVS-11 CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);

G551D-N GAAATTCTTGCTCGTTGAC (SEQ ID NO: 7),
G551D-M GAAATTCTTCGTCGTTGAT (SEQ ID NO: 8), and
IVS-11 CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);

G542X-N GTGTGATTCCACCTTCTCC (SEQ ID NO: 9),
G542X-M GTGTGATTCCACCTTCTCA (SEQ ID NO: 10), and
IVS-11 CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6); and

N1303K-N CACTGTTCATAGGGATCCAAG (SEQ ID NO: 11),
N1303K-M CACTGTTCATAGGGATCCAAC (SEQ ID NO: 12), and
IVS-21 AAGAATGATACAAAGCAGACATG (SEQ ID NO: 13).

It is therefore an object of the invention to provide methods of diagnosing cystic fibrosis. It is a further object of the invention to provide kits useful for diagnosing cystic fibrosis. These and other objects will become apparent by an examination of the detailed description and accompanying claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of a strategy for multiplex amplification of the areas encompassing the most common cystic fibrosis mutations. Approximate location of five common cystic fibrosis mutations is indicated (▲) within the CFTR gene. Multiplex amplification is accomplished using allele specific primer sets. Common primers (CF-508RP, 5′ IVS-11 and 5′ IVS-21) and a mixture selected from five normal (CF-W1468, R553X-N, G551D-N, G542X-N and N1303K-N) and/or five mutant primers (ΔF508, R553X-M, G551D-M, G542X-M and N1303K-M) are used in each PCR reaction. Size of expected PCR products in base pairs is also shown.

Figure 2 shows PCR amplification of normal DNA in

regions encompassing the most common cystic fibrosis mutations. All amplifications were done with either normal (N) or mutant (M) primers corresponding to the ΔF508, G551D, G542X and N1303K regions of the human CFTR gene. Amplifications were done with DNA from normal controls using normal primers (lanes 2,4,6 and 8) or mutant primers for the same regions (lanes 3,5,7 and 9). PCR products are sized relative to markers generated from a Hae III digest of ϕX174 Rf DNA (lane 1). Arrows show location of each PCR product using indicated primers.

Figure 3 shows the detection of common cystic fibrosis mutations by multiplex allele specific polymerase chain reaction (MASPCR). Multiplex amplification of four regions in normal DNA (lanes 2-5) encompassing ΔF508, G551D, G542X and N1303K mutations with normal (N) primers (lanes 2 and 4) or mutant (M) primers (lanes 3 and 5) was carried out. Multiplex amplification using four primer sets was done using separate reactions containing a mixture of either the normal or corresponding mutant primer pairs with DNA from a heterozygote for the ΔF508 mutation (lanes 6 and 7), DNA from a heterozygote for the G542X mutation (lanes 8 and 9), and DNA from a compound heterozygote for the ΔF508 and G542X mutations (lanes 10 and 11), respectively. PCR products are sized relative to markers generated from a Hae III digest of ϕX174 Rf DNA (lanes 1 and 12).

Figure 4 is a graphical representation of the detection of the ΔF508 allele in the CFTR gene by fluorescence-based allele-specific PCR. PCR products were analyzed on a Gene Scanner 362 Fluorescent Fragment Analyzer (Applied Biosystems, Foster City, CA, U.S.A). Panels A-D are graphic representations of fluorescence emission from the reactions with three (Panel A), two (Panel B) or single colors (Panels C and D), displayed by analysis software for comparison of overlapping signals. The third color (red) is not shown. Panels E through G are electrophoretograms showing signal traces through lanes 20, 21 and 22, respectively, of panel A with the different colors displayed separately.

DETAILED DESCRIPTION OF THE INVENTION

Rapid, simple, non-radioactive methods for detection of mutations causing a disease such as cystic fibrosis (CF) are provided by the present invention. In some methods of the present invention, genomic DNA is obtained from a patient suspected of carrying a genetic mutation characteristic of a disease such as CF. Genomic DNA may be extracted by methods described by Poncz, et al., *Hemoglobin* 6: 27-33 (1982) or with an automated extractor (Applied Biosystems, Inc., Foster City, CA). Other methods for extraction of genomic DNA known to those skilled in the art are also encompassed by the present invention.

The gene responsible for cystic fibrosis is the cystic fibrosis transmembrane conductance regulator (CFTR) gene. More than 120 different mutations have been reported in the CFTR gene. Kerem, et al., *Science* 245: 1073-1080 (1989); Zielenski, et al., *Genomics* 10: 214-228 (1991); Dean, et al., *Cell* 61: 863-870 (1990); Cutting, et al., *Nature* 364: 366-369 (1990); Kerem, et al. *Proc. Natl. Acad. Sci. U.S.A.* 87: 8447-8451 (1990) and Cystic Fibrosis Genetic Analysis Consortium, *Am. J. Hum. Genet.* 47: 354-359 (1990). Some common mutations are set forth in Table I.

TABLE I

COMMON CYSTIC FIBROSIS MUTATIONS

Mutation	Amino Acid Change	CFTR Exon	Frequency*
ΔF508	Deletion of Phe-508	10	75.8%
G542X	Gly-542 to stop	11	2.7%
G551D	Gly-551 to Asp	11	3.2%
R553X	Arg-553 to stop	11	1.4%
N1303K	Asn-1303 to Lys	21	1.4%

* Ng, I.S.L., et al., *Human Genet.* 87: 613-617 (1991)

A multiplex allele-specific polymerase chain reaction (MASPCR) can be used to detect mutations such as the mutations of Table I by the appropriate choice of primers. In some embodiments of the present invention a primer strategy such as the strategy set forth in Figure 1 can be used to detect at

least two of the mutations of Table I simultaneously. Primers are designed so that the size of the resulting PCR products differ, thereby facilitating detection. Oligonucleotide primers of the present invention can be synthesized by procedures known to those skilled in the art such as by solid state phosphoramidite synthesis.

In accordance with methods of the present invention at least two primer sets for detecting at least two mutations characteristic of a disease such as cystic fibrosis are selected. In some embodiments of the present invention four primer sets are selected useful for diagnosing four mutations characteristic of cystic fibrosis. In still further preferred embodiments of the present invention five primer sets are selected which are useful for detecting five mutations characteristic of cystic fibrosis. Each primer set is comprised of two primer pairs. A first primer pair is comprised of a primer specific for a normal allele. A second primer pair is comprised of a primer specific for a mutant allele such as an allele specific for cystic fibrosis. The specific primers differ from each other only at their terminal 3′ nucleotide. Thus, for example, the 3′ nucleotide of the specific primer of the first primer pair is specific for the nucleic acid sequence of a normal allele. The 3′ nucleotide of the specific primer of the second primer pair may be specific for the nucleic acid sequence of a cystic fibrosis mutation. Each primer pair further comprises a common primer. Thus, the nucleic acid sequence of the common primer is the same for both primer pairs comprising a primer set. Under proper annealing temperatures and polymerase chain reaction conditions, these primers pairs only direct amplification of their complementary allele. For example, genomic DNA from a patient homozygous for the ΔF508 mutation will be amplified by the second primer pair (mutant specific). The first primer pair, specific for the normal allele will not amplify genomic DNA from a patient homozygous for a mutant allele.

In some preferred embodiments of the present invention, each specific primer is differentially labeled,

resulting in differentially labeled PCR products. Any label known to those skilled in the art which can be easily differentiated clinically are encompassed by the present invention. For example, dyes known to those skilled in the art may be useful to distinguish PCR products based upon color differentiation. In preferred embodiments of the present invention fluorescent dyes such as FAM™ (blue), JOE™ (green), TAMRA™ (yellow) and ROX™ (red) (Applied Biosystems, Inc., Foster City, CA) may be used. Differential labels may be linked to oligonucleotide primers of the present invention by methods known to those skilled in the art, such as by linker molecules. Linker molecules useful in the present invention may be selected from any of a variety of linker molecules available to those skilled in the art, such as a reactive aminohexyl linker (Aminolink). In other embodiments of the present invention differential label may be incorporated during synthesis of the oligonucleotide primers. In still other embodiments of the present invention, PCR products are labeled by differential recognition by a labeled probe or chemical moiety such as a rhodamine coupled antibody. For example, the specific primer for the N1303K mutant allele may be labeled with yellow dye and the N1303K normal allele labeled with blue dye. By detecting a yellow signal, one skilled in the art would be apprised that the patient has a N1303K mutant allele. A blue signal would indicate a normal allele.

Some primer sets useful in the present invention are set forth in Table II.

TABLE II

OLIGONUCLEOTIDE PRIMERS SETS USED FOR MUTATION DETECTION BY MASPCR

Set No.	SEQ. ID NO:	Primer	Primer Type	Sequence	pMoles
1	1	CF-W1468	normal	GGCACCATTAAAGAAAATATCATCTT	15
	2	ΔF508	mutant	GGCACCATTAAAGAAAATATCATTGG	15
	3	CF-508RP	common	TAGTGTGAAGGGTTCATATGCATAAT	15
2	4	R553X-N	normal	CTAAAGAAATTCTTGCTCG	10
	5	R553X-M	mutant	CTAAAGAAATTCTTGCTCA	10
	6	5'IVS-11	common	CAACTGTGGTTAAAGCAATAGTGT	20
3	7	G551D-N	normal	GAAATTCTTGCTCGTTGAC	5
	8	G551D-M	mutant	GAAATTCTTGCTCGTTGAT	5
	6	5'IVS-11	common	CAACTGTGGTTAAAGCAATAGTGT	20
4	9	G542X-N	normal	GTGTGATTCCACCTTCTCC	20
	10	G542X-M	mutant	GTGTGATTCCACCTTCTCA	20
	6	5'IVS-11	common	CAACTGTGGTTAAAGCAATAGTGT	20
5	11	N1303K-N	normal	CACTGTTCATAGGGATCCAAG	40
	12	N1303K-M	mutant	CACTGTTCATAGGGATCCAAC	40
	13	5'IVS-21	common	AAGAATGATACAAAGCAGACATG	40

"N" and "M" indicate normal and mutant primers, respectively. Common primer indicates primers having a sequence common to both primer pairs. Normal and mutant indicate primers having a sequence specific for a normal or mutant allele, respectively. Bold letters indicate single-base mutations. Amount (pMoles) of each primer used in multiplex reactions per primer pair is also indicated. Sequences are provided in 5' to 3' direction.

Other primer sets useful to detect a particular disease such as cystic fibrosis can be identified using methods known to those skilled in the art. The first member in each set is the specific primer for a normal allele, the second member of each set is the specific primer for the corresponding mutant allele, and the third member of each set is the common primer included in each primer pair. The names of the primers generally correspond to the mutation they are designed to detect. For example, N1303K-N and N1303K-M are the primers useful for amplification of the N1303K normal and N1303K mutant alleles, respectively.

At least two mutant alleles can be detected simultaneously by methods of the present invention. It is encompassed by some embodiments of the present invention to perform two polymerase chain reactions per diagnosis. In one PCR reaction mixture, primer pairs for normal alleles from each primer set are used. In a second reaction mixture, all primer pairs for mutant alleles from each primer set are used. Thus each polymerase chain reaction in such a diagnostic test causes amplification of genomic DNA using either primers specific for mutant or normal alleles, i.e. half of each primer set per reaction, an entire primer set per diagnosis. Resulting PCR products are run in parallel on gels to detect the presence or absence of bands. For example, diagnosis of cystic fibrosis is accomplished in some embodiments of the present invention, by comparison of normal and mutant polymerase chain reaction products. Figure 3, for example, shows five, two PCR diagnoses using four primer sets each. In each diagnosis, one PCR reaction was performed with primers specific for normal alleles (lanes 2,4,6,8 and 10) and one PCR reaction was performed with primers specific for mutant alleles (lanes 3,5,7,9 and 11). PCR products run in parallel on a gel confirmed the following genotypes, a ΔF508 heterozygote (comparison of lanes 6 and 7), a G542X heterozygote (lanes 8 and 9) and a ΔF508/G542X compound heterozygote (lanes 10 and 11). Diagnosis, may be used in the context of the present invention to encompass a procedure whereby two or more primer sets are amplified and interpreted

in order to determine the presence or absence of selected normal and mutant alleles in a particular genomic DNA sample. As has been exemplified above, a diagnosis may encompass one or more polymerase chain reactions.

In preferred embodiments of the present invention both members of each primer pair of at least two primer sets are used simultaneously in a single polymerase chain reaction which is run on a single lane of a gel. Differential labels, as described above, are useful herein for distinguishing polymerase chain reaction products, especially those having similar mobilities. Thus PCR products can be distinguished by mobility and label. In addition, labels, such as fluorescent labels may be particularly amenable to automated methods.

In some preferred embodiments of the present invention at least two of the primer sets provided in Table II are selected. It is still more preferred in some embodiments of the present invention to select all of the primer sets of Table II. In some embodiments of the present invention the primer sets 1 (ΔF508/CF-W1468), 3 (G551D-N/G551D-M), 4 (G542X-N/G542X-M) and 5 (N1303K-N/N1303K-M) are selected. The primer sets 1 (ΔF508/CF-W1468), 3 (G551D-N/G551D-M) and 4 (G542X-N/G542X-M) may be selected in accordance with some embodiments of the present invention. In still other embodiments of the present invention the primer sets 1 (ΔF508/CF-W1468) and 3 (G551D-N/G551D-M) are selected.

Kits are also provided by the present invention comprising four dNTPs and at least two primer sets selected from the primer sets provided in Table II.

The following examples are illustrative, but should not be construed as limiting the present invention.

EXAMPLES

EXAMPLE 1

DNA Samples

DNA samples were obtained from normal controls and patients either homozygous or heterozygous for the common CF mutations, ΔF508, G551D, R553X, G542X and N1303K. Genomic DNA was extracted using protocols previously described in Poncz,

M.D., et al., *Hemoglobin* 6: 27-33 (1982) or with an automated extractor (ABI, Foster City, CA). Genotypes were confirmed either by manual DNA sequence analysis or by allele-specific oligonucleotide hybridization.

EXAMPLE 2

Primer Synthesis

Unlabelled oligonucleotide primers were prepared by standard phosphoramidite chemistry. Caruthers, M.H., et al., *Methods in Enzymol.* 154: 287-313 (1987). Oligonucleotide primers were prepared for fluorescent labeling following standard phosphoramidite chemistry preparation by attachment of a reactive aminohexyl linker group (Aminolink) to the 5′ end of the primer. Draper, D. and L.E. Gold, *Biochemistry* 19: 1774-1781 (1980). Following cleavage from the solid support, and deprotection, salts were removed from the crude primer and the oligonucleotide was dissolved in 0.5M $NaHCO_3/Na_2CO_3$, pH 9.0 buffer. Fluorescent dye-N-hydroxyl succinimide esters were dissolved in DMSO and added to oligonucleotide primer aliquots. The reaction was allowed to proceed at room temperature for 2-24 hours. ABI 370 user bulletin (1989). The dye labeled primer was then removed from excess reactants via high performance liquid chromatography purification. Sequences of the oligonucleotide primers are as provided in Table II.

EXAMPLE 3

Polymerase Chain Reaction

PCR was performed according to methods previously described by Saiki, R.K., et al., *Science* 239: 487-489 (1988). Reaction mixtures (25μl) contained 100ng of genomic DNA, 1.5 μM of each dNTP, the common primers (CF-508RP, 5′ IVS-11 and 5′ IVS-21) and normal and/or mutant primers in a buffer containing 6.7mM $MgCl_2$, 16.6 mM $(NF_4)_2SO_4$, 5.0 μM βME, 6.8 mM EDTA, 67.0 mM Tris HCl pH 8.8, 10% (v/v) DMSO. The mixture was heated at 95°C for 5 minutes to denature the DNA, and then quickly chilled on ice. Taq DNA polymerase (1.5 U, Perkin Elmer, Norwalk, CT) was added before overlaying the samples with 25 μl

of mineral oil. The samples were then subjected to 30 cycles on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) with denaturation at 95°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute. The last cycle had a 5 minute extension at 72°C. Approximately 15μl of the PCR product were then analyzed following electrophoresis on a 3% (w/v) agarose (NuSieve GTG) gel.

EXAMPLE 4

Selectivity of Oligonucleotide Primers

Differently sized PCR products were produced based upon the strategy illustrated in Figure 1. Unlabelled normal and mutant PCR primers were synthesized so their terminal 3' nucleotide corresponds to either a normal or mutant sequence. Primers were tested with normal and available mutant DNA samples under stringent PCR conditions to ensure that selective amplification occurred with either normal or mutant primers. Normal and mutant products were scored by the presence or absence of correctly sized bands following electrophoresis on agarose gel. PCR was performed in accordance with Example 3.

Separate amplifications using normal primers for each of the four regions in normal DNA gives single bands of expected size (Figure 2, lanes 2,4,6 and 8). Multiplex PCR using normal primers for 4 of the 5 regions in normal DNA also yields the expected combined band pattern (Figure 3, lanes 2 and 4), while no amplification products were seen with the mutant primer set (Figure 2, lanes 3 and 5).

EXAMPLE 5

Diagnosis of Cystic Fibrosis Using MASPCR

Different known CF genotypes were used to test the accuracy of the method. Unlabeled mutant and normal PCR products G551D and R553X are too similar in size to be resolved using this multiplex method. Thus, in order to test the primers four separate PCR reactions were performed in accordance with Example 3. Four mutations were tested by doing two separate PCR reactions. Each reaction contained three

common primers; CF-508RP, 5′ IVS-11 and 5′ IVS-21; and either four mutant or four normal primers for the ΔF508, G551D, G542X and N1303K sequences. Data are shown in Figure 3 confirming the following genotypes: ΔF508 heterozygote (lanes 6 and 7), G542X heterozygote (lanes 8 and 9) and a ΔF508/G542X compound heterozygote (lanes 10 and 11). The R553X primers were tested with two additional PCR reactions (with normal and mutant primers). (Data not shown).

EXAMPLE 6

Diagnosis of the ΔF508 Cystic Fibrosis Deletion using MASPCR and Fluorescently Labeled Primers

The normal specific primer (CF-W1468) is labeled with a blue tag and the mutant specific primer (CF-ΔF508) with a green tag. The common primer (CF-508RP) was unlabeled, and internal size standards were labeled with a red tag (not shown). PCR was performed and the PCR product was then analyzed following electrophoresis on a 2% (w/v) agarose (NuSieve GTG) gel using a multi-line argon ion laser such as a GENE SCANNER™ (Applied Biosystems, Inc. Foster City, CA) to detect the fluorescently labeled PCR products. Results are provided in Figure 4. In panel A, a graphic recreation of the real-time fluorescence emission from the reactions during electrophoresis is shown, displaying two colors (a third not shown). Panel B excludes the red fluorescence signal (not shown) from the marker fragments, panel C displays only the blue signal and panel D shows only the green signal. The lower band in each lane represents excess primer not incorporated into the PCR reaction. In panels C and D, both blue and green signals indicates that both normal and ΔF508 specific primers were included in each reaction. The reaction in lane 20 is from a normal control consistent with the incorporation of only blue-labeled wild-type primers into a PCR product 79 bp in size (panel C). Lane 21 contains a reaction using genomic DNA from an individual homozygous for the ΔF508 mutation, showing appropriate incorporation of only green-labeled mutant primers into a PCR product 76 bp in length (panel D). The sample in

lane 22 contains DNA from a heterozygote for the ΔF508 deletion, yielding amplification with both primers, and resulting in both blue 79 bp (panel C) and green 76 bp (panel D) bands. Panels E, F and G show electrophoretograms of lanes 20, 21, and 22, respectively, of panel A, which represents signal traces within each lane and indicate the relative positions and areas of the bands.

EXAMPLE 7

Diagnosis of Four Common Cystic Fibrosis Deletions using Fluorescently Labeled Primers

Diagnosis is carried out as provided in Example 5, except that both normal and mutant allele primer sets are combined in a single PCR reaction mixture. Each reaction contains three common primers; CF-508RP, 5′ IVS-11 and 5′ IVS-21; four mutant and four normal primers for the ΔF508, G551D, G542X and N1303K sequences. Primers are labeled as follows: CF-W1468 (blue), ΔF508 (green), G542X-N (yellow), G542X-M (red), G551D-N (blue), G551D-M (green) N1303K-N (yellow) and N1303K-M (red). A fluorescently labeled marker lane is run in a separate lane to facilitate sizing PCR products. The PCR product is then analyzed following electrophoresis on a 3% (w/v) agarose (NuSieve GTG) gel using a multi-line argon ion laser such as a GENE SCANNER™ (Applied Biosystems, Inc. Foster City, CA) to detect the fluorescently labeled PCR products. Multicolored bands indicate heterozygosity for an allele, while single colored bands indicate homozygosity for an allele.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Fortina, Paolo
Surrey, Saul

(ii) TITLE OF INVENTION: DIAGNOSIS OF CYSTIC FIBROSIS USING ALLELE SPECIFIC MULTIPLEX POLYMERASE CHAIN REACTIONS

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz and Norris
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(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: U.S.A.
(F) ZIP: 19103

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Johnson, Philip S.
(B) REGISTRATION NUMBER: 27,200
(C) REFERENCE/DOCKET NUMBER: CH-0224

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-568-3100
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCACCATTA AAGAAAATAT CATCTT 26

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGCACCATTA AAGAAAATAT CATTGG 26

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TAGTGTGAAG GGTTCATATG CATAAT 26

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CTAAAGAAAT TCTTGCTCG 19

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTAAAGAAAT TCTTGCTCA 19

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAACTGTGGT TAAAGCAATA GTGT 24

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GAAATTCTTG CTCGTTGAC 19

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GAAATTCTTG CTCGTTGAT 19

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GTGTGATTCC ACCTTCTCC 19

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTGTGATTCC ACCTTCTCA 19

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CACTGTTCAT AGGGATCCAA G 21

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CACTGTTCAT AGGGATCCAA C 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AAGAATGATA CAAAGCAGAC ATG 23

What is claimed is:

1. A method of diagnosing cystic fibrosis comprising the steps of:

obtaining genomic DNA from a patient suspected of carrying a genetic mutation characteristic of the phenotype cystic fibrosis;

selecting at least two primer sets for detecting at least two mutations characteristic of cystic fibrosis, each set being comprised of two primer pairs, a first primer pair comprising a specific primer for a normal allele, and a second primer pair comprising a specific primer for a mutant allele, each pair further comprising a common primer;

performing a polymerase chain reaction using said genomic DNA and said at least two primer sets whereby primer pairs comprising a specific primer for a normal allele are used simultaneously and primer pairs comprising a specific primer for a mutant allele are used simultaneously; and

detecting two or more polymerase chain reaction products whereby the detection of a polymerase chain reaction product of a specific primer for a mutant allele indicates the likelihood that said patient carries a mutation characteristic of the phenotype cystic fibrosis.

2. The method of claim 1 wherein at least four primer sets are selected.

3. The method of claim 1 wherein at least five primer sets are selected.

4. The method of claim 1 wherein the step of selecting at least two primer sets further comprises selecting primer sets in which each specific primer is differentially labeled.

5. The method of claim 4 wherein the step of performing a polymerase chain reaction further comprises using said genomic DNA and all differentially labeled primer sets simultaneously.

6. The method of claim 1 wherein the step of selecting further comprises selecting primer sets comprising a first, second and third member, the first member being a specific primer for a normal allele, the second member being a specific primer for a mutant allele, and the third member being a common allele from the group consisting of:

CF-W1468	GGCACCATTAAAGAAAATATCATCTT (SEQ ID NO: 1),
Δf508	GGCACCATTAAAGAAAATATCATTGG (SEQ ID NO: 2), and
CF-508RP	TAGTGTGAAGGGTTCATATGCATAAT (SEQ ID NO: 3);
R553X-N	CTAAAGAAATTCTTGCTCG (SEQ ID NO: 4),
R553X-M	CTAAAGAAATTCTTGCTCA (SEQ ID NO: 5), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);
G551D-N	GAAATTCTTGCTCGTTGAC (SEQ ID NO: 7),
G551D-M	GAAATTCTTCGTCGTTGAT (SEQ ID NO: 8), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);
G542X-N	GTGTGATTCCACCTTCTCC (SEQ ID NO: 9),
G542X-M	GTGTGATTCCACCTTCTCA (SEQ ID NO: 10), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6); and
N1303K-N	CACTGTTCATAGGGATCCAAG (SEQ ID NO: 11),
N1303K-M	CACTGTTCATAGGGATCCAAC (SEQ ID NO: 12), and
IVS-21	AAGAATGATACAAAGCAGACATG (SEQ ID NO: 13).

7. The method of claim 1 wherein the polymerase chain reaction is performed by using said genomic DNA and the primer sets:

CF-W1468	GGCACCATTAAAGAAAATATCATCTT (SEQ ID NO: 1),
Δf508	GGCACCATTAAAGAAAATATCATTGG (SEQ ID NO:2), and
CF-508RP	TAGTGTGAAGGGTTCATATGCATAAT (SEQ ID NO: 3);
R553X-N	CTAAAGAAATTCTTGCTCG (SEQ ID NO: 4),
R553X-M	CTAAAGAAATTCTTGCTCA (SEQ ID NO: 5), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);
G551D-N	GAAATTCTTGCTCGTTGAC (SEQ ID NO: 7),
G551D-M	GAAATTCTTCGTCGTTGAT (SEQ ID NO: 8), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);
G542X-N	GTGTGATTCCACCTTCTCC (SEQ ID NO: 9),
G542X-M	GTGTGATTCCACCTTCTCA (SEQ ID NO: 10), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6); and
N1303K-N	CACTGTTCATAGGGATCCAAG (SEQ ID NO: 11),
N1303K-M	CACTGTTCATAGGGATCCAAC (SEQ ID NO: 12), and
IVS-21	AAGAATGATACAAAGCAGACATG (SEQ ID NO: 13);

each of said primer sets being comprised of a first, second and third member, the first member being a specific primer for a normal allele, the second member being a specific primer for a mutant allele, and the third member being a common allele.

8. The method of claim 7 wherein the polymerase chain reaction is performed by using said genomic DNA and said primer sets, the said first and second members of each primer set further being differentially labelled.

9. The method of claim 8 wherein the polymerase chain reaction is performed by using said genomic DNA and all differentially labeled primer sets simultaneously.

10. The method of claim 1 wherein the polymerase chain reaction is performed by using said genomic DNA and the primer sets:

CF-W1468	GGCACCATTAAAGAAAATATCATCTT (SEQ ID NO: 1),
Δf508	GGCACCATTAAAGAAAATATCATTGG (SEQ ID NO: 2), and
CF-508RP	TAGTGTGAAGGGTTCATATGCATAAT (SEQ ID NO: 3);
G551D-N	GAAATTCTTGCTCGTTGAC (SEQ ID NO: 7),
G551D-M	GAAATTCTTCGTCGTTGAT (SEQ ID NO: 8), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);
G542X-N	GTGTGATTCCACCTTCTCC (SEQ ID NO: 9),
G542X-M	GTGTGATTCCACCTTCTCA (SEQ ID NO: 10), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6); and
N1303K-N	CACTGTTCATAGGGATCCAAG (SEQ ID NO: 11),
N1303K-M	CACTGTTCATAGGGATCCAAC (SEQ ID NO: 12), and
IVS-21	AAGAATGATACAAAGCAGACATG (SEQ ID NO: 13);

each of said primer sets being comprised of a first, second and third member, the first member being a specific primer for a normal allele, the second member being a specific primer for a mutant allele, and the third member being a common allele.

11. The method of claim 10 wherein the polymerase chain reaction is performed by using said genomic DNA and said primer sets, the said first and second members of each primer set further being differentially labelled.

12. The method of claim 11 wherein the polymerase chain reaction is performed by using said genomic DNA and all differentially labeled primer sets simultaneously.

13. The method of claim 1 wherein the polymerase chain reaction is performed by using said genomic DNA and the primer sets:

CF-W1468	GGCACCATTAAAGAAAATATCATCTT (SEQ ID NO: 1),
Δf508	GGCACCATTAAAGAAAATATCATTGG (SEQ ID NO: 2), and
CF-508RP	TAGTGTGAAGGGTTCATATGCATAAT (SEQ ID NO: 3);
G551D-N	GAAATTCTTGCTCGTTGAC (SEQ ID NO: 7),
G551D-M	GAAATTCTTCGTCGTTGAT (SEQ ID NO: 8), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6); and
G542X-N	GTGTGATTCCACCTTCTCC (SEQ ID NO: 9),
G542X-M	GTGTGATTCCACCTTCTCA (SEQ ID NO: 10), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6).

each of said primer sets being comprised of a first, second and third member, the first member being a specific primer for a

normal allele, the second member being a specific primer for a mutant allele, and the third member being a common allele.

14. The method of claim 13 wherein the polymerase chain reaction is performed by using said genomic DNA and said primer sets, the said first and second members of each primer set further being differentially labelled.

15. The method of claim 14 wherein the polymerase chain reaction is performed by using said genomic DNA and all differentially labeled primer sets simultaneously.

16. The method of claim 1 wherein the polymerase chain reaction is performed by using said genomic DNA and the primer sets:

CF-W1468	GGCACCATTAAAGAAAATATCATCTT (SEQ ID NO: 1),
Δf508	GGCACCATTAAAGAAAATATCATTGG (SEQ ID NO: 2), and
CF-508RP	TAGTGTGAAGGGTTCATATGCATAAT (SEQ ID NO: 3); and
G551D-N	GAAATTCTTGCTCGTTGAC (SEQ ID NO: 7),
G551D-M	GAAATTCTTCGTCGTTGAT (SEQ ID NO: 8), and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6).

each of said primer sets being comprised of a first, second and third member, the first member being a specific primer for a normal allele, the second member being a specific primer for a mutant allele, and the third member being a common allele.

17. The method of claim 16 wherein the polymerase chain reaction is performed by using said genomic DNA and said primer sets, the said first and second members of each primer set further being differentially labelled.

18. The method of claim 17 wherein the polymerase chain reaction is performed by using said genomic DNA and all differentially labeled primer sets simultaneously.

19. A kit comprising four dNTPs and at least two primer sets selected from the group consisting of:

CF-W1468	GGCACCATTAAAGAAAATATCATCTT (SEQ ID NO: 1),	
Δf508	GGCACCATTAAAGAAAATATCATTGG (SEQ ID NO: 2),	and
CF-508RP	TAGTGTGAAGGGTTCATATGCATAAT (SEQ ID NO: 3);	
R553X-N	CTAAAGAAATTCTTGCTCG (SEQ ID NO: 4),	
R553X-M	CTAAAGAAATTCTTGCTCA (SEQ ID NO: 5),	and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);	
G551D-N	GAAATTCTTGCTCGTTGAC (SEQ ID NO: 7),	
G551D-M	GAAATTCTTCGTCGTTGAT (SEQ ID NO: 8),	and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);	
G542X-N	GTGTGATTCCACCTTCTCC (SEQ ID NO: 9),	
G542X-M	GTGTGATTCCACCTTCTCA (SEQ ID NO: 10),	and
IVS-11	CAACTGTGGTTAAAGCAATAGTGT (SEQ ID NO: 6);	and
N1303K-N	CACTGTTCATAGGGATCCAAG (SEQ ID NO: 11),	
N1303K-M	CACTGTTCATAGGGATCCAAC (SEQ ID NO: 12),	and
IVS-21	AAGAATGATACAAAGCAGACATG (SEQ ID NO: 13).	

20. A method of diagnosing a disease characterized by multiple allelic mutations comprising the steps of:

obtaining genomic DNA from a patient suspected of carrying a genetic mutation characteristic of the disease;

selecting at least two primer sets for detecting at least two mutations characteristic of the disease, each set being comprised of a specific primer for a normal allele, a specific primer for a mutant allele, and a common primer;

performing a polymerase chain reaction by using said genomic DNA and said at least two primer sets whereby primer pairs comprising a specific primer for a normal allele are used simultaneously and primer pairs comprising a specific primer for a mutant allele are used simultaneously; and

detecting one or more polymerase chain reaction products wherein detection of a polymerase chain reaction product of a specific primer for a mutant allele indicates the likelihood that said patient carries a mutation characteristic of the disease.

21. The method of claim 20 wherein at least 4 primer sets are selected.

22. The method of claim 20 wherein at least 5 primer

sets are selected.

23. The method of claim 20 wherein the step of selecting at least two primer sets further comprises selecting primer sets in which each specific primer is differentially labelled.

24. The method of claim 23 wherein the step of performing a polymerase chain reaction further comprises using said primer sets simultaneously.

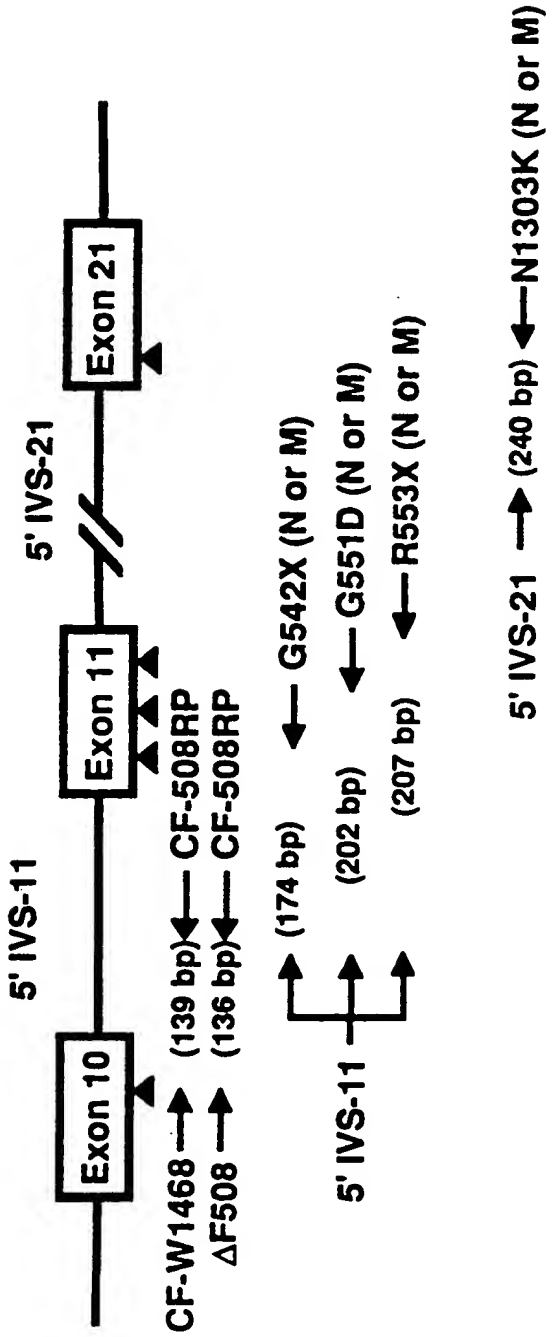

Exon 21
5' IVS-21
Exon 11
5' IVS-11
Exon 10
CF-W1468
ΔF508
(139 bp)
(136 bp)
CF-508RP
CF-508RP
(174 bp)
G542X (N or M)
5' IVS-11
(202 bp)
G551D (N or M)
(207 bp)
R553X (N or M)
5' IVS-21
(240 bp)
N1303K (N or M)

FIG. 1

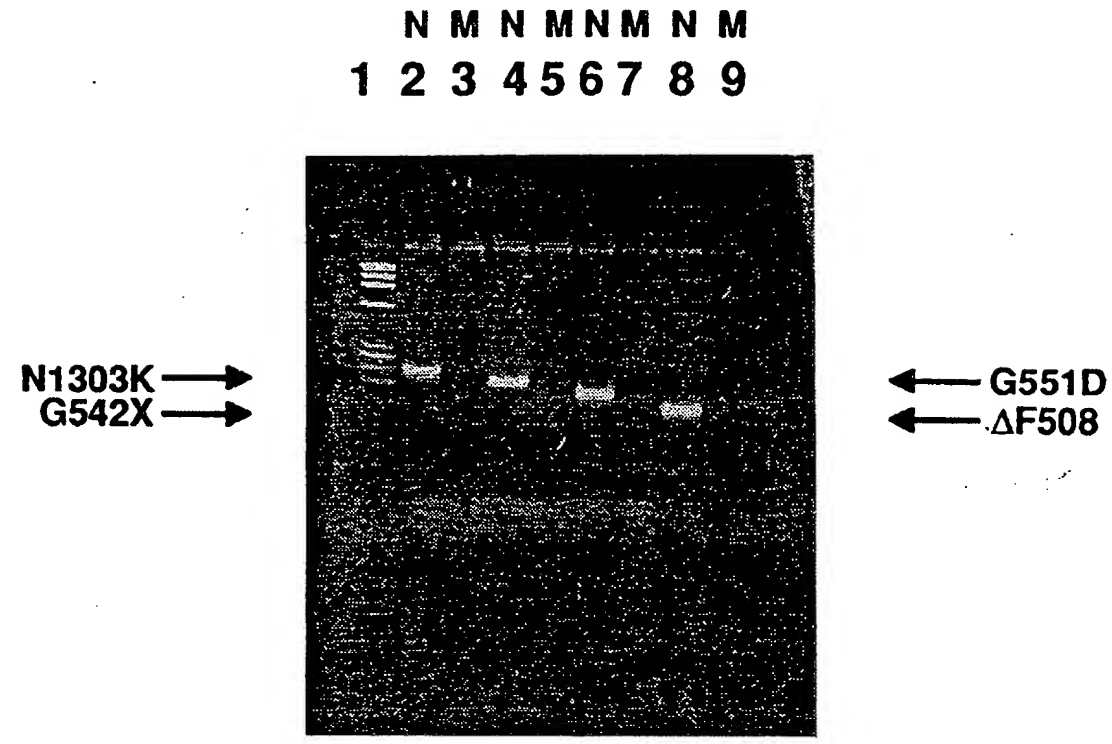

N M N M N M N M
1 2 3 4 5 6 7 8 9
N1303K
G542X
G551D
ΔF508

FIG.2

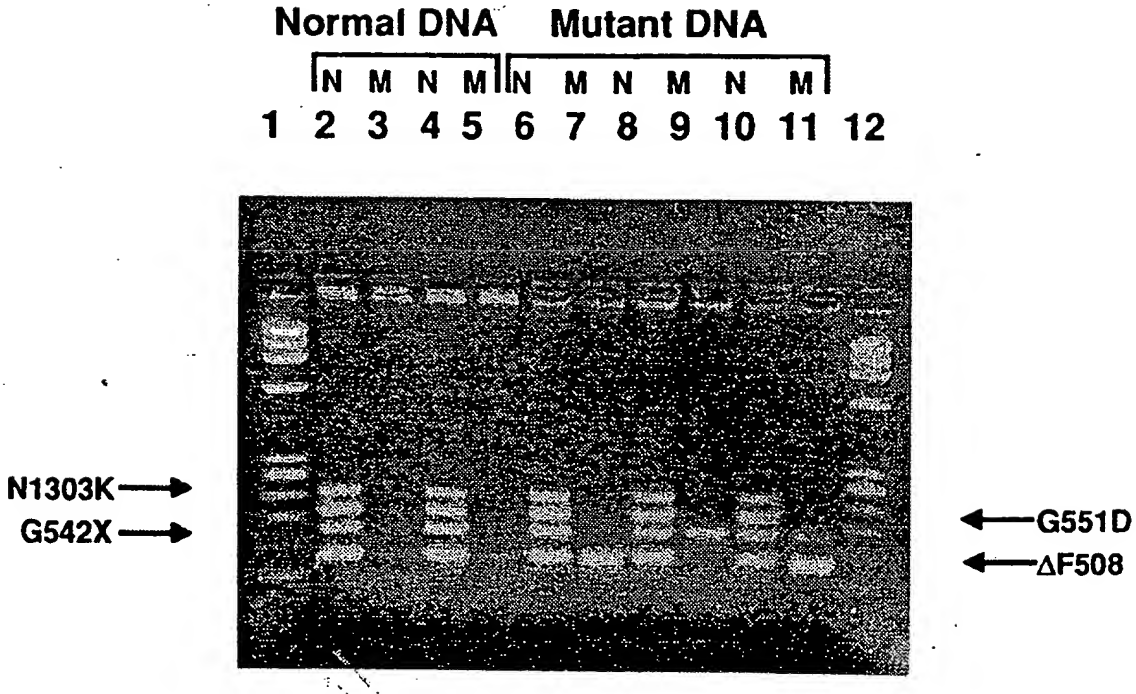

Normal DNA
Mutant DNA
N M N M N M N M N M
1 2 3 4 5 6 7 8 9 10 11 12
N1303K
G542X
G551D
ΔF508

FIG.3

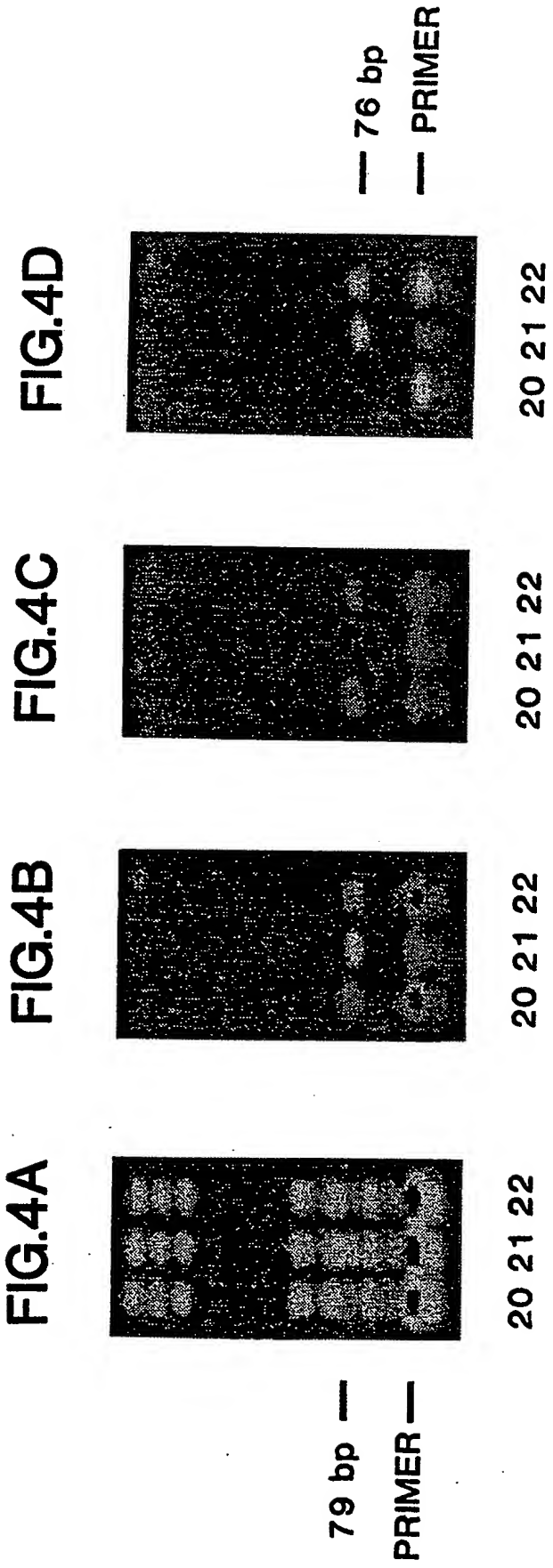


FIG.4A
FIG.4B
FIG.4C
FIG.4D
79 bp
PRIMER
76 bp
PRIMER
20 21 22
20 21 22
20 21 22
20 21 22

FIG. 4E

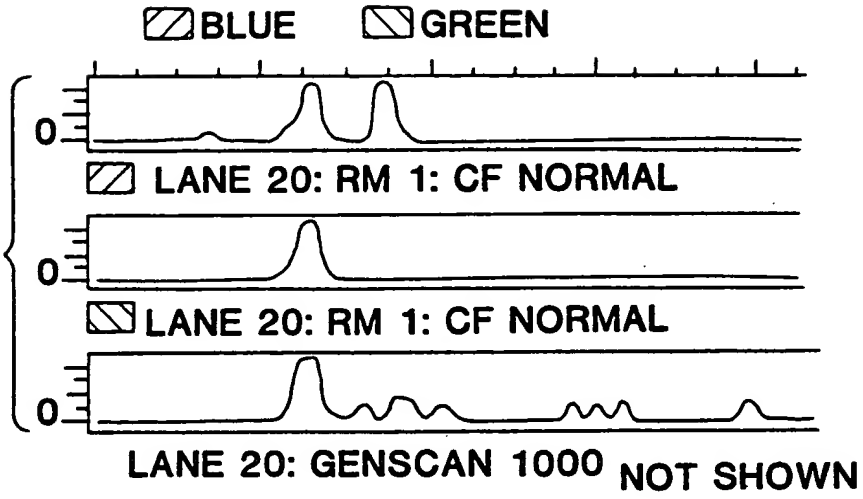


FIG. 4F

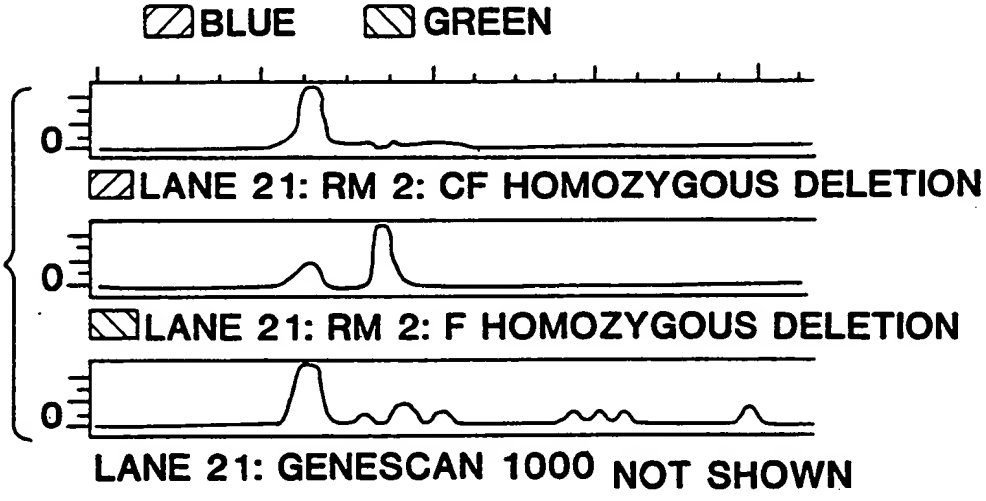
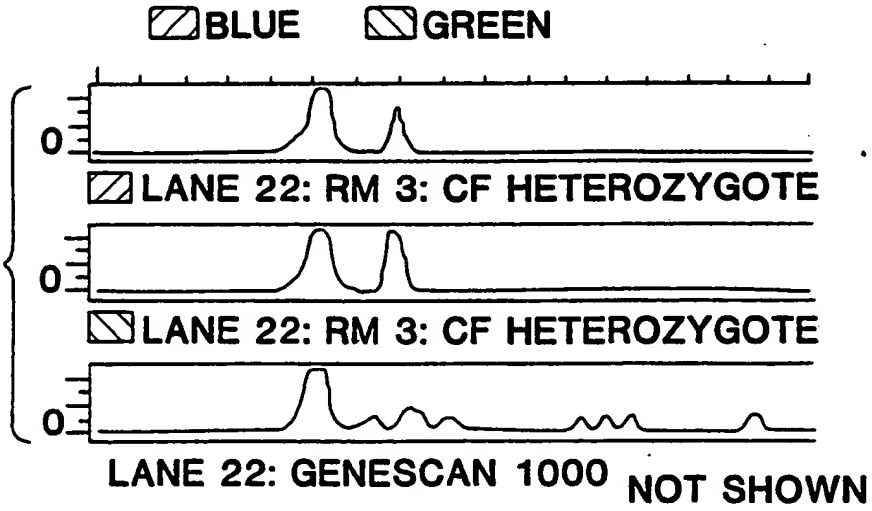


FIG. 4G



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02259

A. CLASSIFICATION OF SUBJECT MATTER
IPC(5) :C12P 19/34; C12Q 1/68
US CL :435/6, 91
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nucleic Acids Research, Volume 17, No. 22, issued 1989, M. Becker-André et al, "Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATTY)", pages 9437-9446, especially Figures 1-3.	1-24
Y	Clinical Chemistry, Volume 36, No. 10, issued 1990, T. W. Prior et al, "A Model for Molecular Screening of Newborns: Simultaneous Detection of Duchenne/Becker Muscular Dystrophies and Cystic Fibrosis", pages 1756-1759, especially Table 1 and Figures 1-3.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
11 June 1993

Date of mailing of the international search report
17 JUN 1993

Name and mailing address of the ISA/US
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Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONA SEARCH REPORT

International application No.
PCT/US93/02259

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,855,225 (Fung et al) 08 August 1989, Abstract, col. 5, lines 12-28, col. 11, lines 67-68, and col. 12, lines 1-22.	4, 5, 8, 9, 11, 12, 14, 15, 17, 18, 23, 24
Y	Clinical Chemistry, Volume 37, No. 5, issued 1991, K. J. Friedman et al, "Detecting Multiple Cystic Fibrosis Mutations by Polymerase Chain Reaction-Mediated Site-Directed Mutagenesis", pages 753-755, especially Figures 1 and 3.	1-24
Y	Nucleic Acids Research, Volume 16, No. 23, issued 1988, J. S. Chamberlain et al, "Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification", pages 11141-11156, especially Table 1 and Figure 3.	1-24
Y	EP, A, 0,297,379 (Molecular Diagnostics, Inc.) 04 January 1989, col. 8, lines 13-33.	19

International application No.
PCT/US93/02259

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, CA SEARCH, BIOSIS, N-GENESEQ, GENBANK, GENBANK-NEW, UEMBL, EMBL-NEW, search terms: cystic fibrosis, polymerase chain reaction, allele, specific, amplification, nucleic acid, multiplex, sequences